



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>4</sup> :</b> <b>C12N 9/50, 15/00, C12P 21/02</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 89/ 06279</b> <b>(43) International Publication Date:</b> 13 July 1989 (13.07.89)
<b>(21) International Application Number:</b> PCT/DK89/00002 <b>(22) International Filing Date:</b> 6 January 1989 (06.01.89) <b>(31) Priority Application Number:</b> 64/88 <b>(32) Priority Date:</b> 7 January 1988 (07.01.88) <b>(33) Priority Country:</b> DK  <b>(71) Applicant:</b> NOVO INDUSTRI A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).  <b>(72) Inventors:</b> HASTRUP, Sven ; Frederiksborgvej 10, 3.th, DK-2400 København NV (DK). BRANNER, Sven ; Ved Smedebakken 7A, DK-2800 Lyngby (DK). NORRIS, Fanny ; Ahlmanns Allé 34, DK-2900 Hellerup (DK). PETERSEN, Steffen, B. ; Sneppehøj 15, DK-2750 Ballerup (DK). NØRSKOV-LAURIDSEN, Leif ; Birkevej 10, DK-4600 Køge (DK). JENSEN, Villy, Johannes ; Skovkilden 6, DK-2880 Bagsværd (DK). AASLYNG, Dorrit ; Fyrren 8, Svogerslev, DK-4000 Roskilde (DK).		<b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> MUTATED SUBTILISIN GENES		
<b>(57) Abstract</b> <p>The present invention relates to mutations of the subtilisin gene, some of which result in changes in the chemical characteristics of subtilisin enzyme. Mutations are created at specific nucleic acids of the subtilisin gene and, in various specific embodiments, the mutant enzymes possess altered chemical properties including, but not limited to, increased stability to oxidation, augmented proteolytic activity, and improved washability. The present invention also relates, but is not limited to, the amino acid and DNA sequences for two proteases derived from <i>Bacillus lentus</i> variants, subtilisin 147 and subtilisin 309, as well as mutations of these genes and the corresponding mutant enzymes.</p>		

Best Available Copy

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark			US	United States of America

## Mutated subtilisin genes.

TABLE OF CONTENTS

	<u>Page</u>
5	
1. Field of the Invention.....	3
2. Background of the Invention.....	3
2.1. <u>Bacillus</u> Proteases.....	3
2.2. Subtilisin.....	3
10 2.3. Industrial Applications of Subtilisins.....	5
3. Summary of the Invention.....	6
3.1 Abbreviations.....	6
4. Description of the Figures.....	7
5. Detailed Description of the Invention.....	8
15 5.1. Chemical Structure of Known Subtilisins and of Subtilisin 147 and 309.....	9
5.2. Methods of Producing Mutations in the Subtilisin Gene.....	17
5.2.1. Cloning a Subtilisin Gene.....	18
20 5.2.2. Generation of Random Mutations in the Subtilisin Gene.....	18
5.2.3. Generation of Site-Directed Mutations in the Subtilisin Gene...	22
5.3. Expression of Subtilisin Mutants.....	23
25 5.4. Screening of Mutant Subtilisins.....	24
6. Example: Site-Specific Mutation of the Subtilisin Gene Generates Mutants with Useful Chemical Characteristics.....	26
6.1. Materials and Methods.....	26
30 6.1.1. Bacterial Strains.....	26
6.1.2. Plasmids.....	26
6.1.3. Purification of Subtilisins.....	27

-2-

	6.1.4. Oligonucleotide Synthesis.....	29
	6.1.5. Determination of Oxidation Stability.....	29
	6.1.6. Assay for Proteolytic Activity.....	29
5	6.1.7. Assay for Washability.....	31
	6.1.8. Assay for Thermostability.....	32
	6.2. Results.....	32
	6.2.1. Cloning of the Subtilisin 309 and 147 Genes.....	32
10	6.2.2. Generation of Site-Specific Mutations of the Subtilisin 309 Gene.....	36
	6.2.3. Expression of Mutant Subtilisins...	40
	6.2.4. Oxidation Stability of Mutant Subtilisins.....	40
15	6.2.5. Proteolytic Activity of Mutant Subtilisins.....	41
	6.2.6. Washability of Mutant Subtilisins...	42
	6.2.7. Thermostability of Mutant Subtilisins.....	43
20	6.3. Discussion.....	44

25

30

35

-3-

## 1. FIELD OF THE INVENTION

The present invention relates to mutations of the subtilisin gene which result in changes in the chemical characteristics of subtilisin enzyme. Mutations at specific nucleic acids of the subtilisin gene result in amino acid substitutions and consequently, altered enzyme function. Some of these mutant enzymes exhibit physical properties advantageous to industrial applications, particularly in the detergent industry, providing subtilisin which is more stable to oxidation, possesses greater protease activity, and exhibits improved washability.

## 2. BACKGROUND OF THE INVENTION

### 2.1. BACILLUS PROTEASES

Enzymes cleaving the amide linkages in protein substrates are classified as proteases, or (interchangeably) peptidases (See Walsh, 1979, Enzymatic Reaction Mechanisms. W.H. Freeman and Company, San Francisco, Chapter 3). Bacteria of the Bacillus species secrete two extracellular species of protease, a neutral, or metalloprotease, and an alkaline protease which is functionally a serine endopeptidase, referred to as subtilisin. Secretion of these proteases has been linked to the bacterial growth cycle, with greatest expression of protease during the stationary phase, when sporulation also occurs. Joliffe et al. (1980, J. Bacterial 141:1199-1208) has suggested that Bacillus proteases function in cell wall turnover.

### 2.2. SUBTILISIN

A serine protease is an enzyme which catalyzes the hydrolysis of peptide bonds, in which there is an essential serine residue at the active site (White, Handler, and

-4-

Smith, 1973 "Principles of Biochemistry," Fifth Edition, McGraw-Hill Book Company, NY, pp. 271-272).

5 The serine proteases have molecular weights in the 25,000 to 30,000 range. They are inhibited by diisopropyl-fluorophosphate, but in contrast to metalloproteases, are resistant to ethylenediamine-tetra acetic acid (EDTA) (although they are stabilized at high temperatures by calcium ion). They hydrolyze simple terminal esters and are similar in activity to eukaryotic chymotrypsin, also a serine protease. The alternative term, alkaline protease, 10 reflects the high pH optimum of the serine proteases, from pH 9.0 to 11.0 (for review, see Priest, 1977, Bacteriological Rev. 41:711-753).

A subtilisin is a serine protease produced by Gram-positive bacteria or fungi. A wide variety of subtilisins 15 have been identified, and the amino acid sequences of at least eight subtilisins have been determined. These include six subtilisins from Bacillus strains, namely, subtilisin 168, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin amylosacchariticus, and mesentericopeptidase 20 (Kurihara et al., 1972, J. Biol. Chem. 247:5629-5631; Stahl and Ferrari, 1984, J. Bacteriol. 158: 411-418; Vasantha et al., 1984, J. Bacteriol. 159:811-819, Jacobs et al., 1985, Nucl. Acids Res. 13:8913-8926; Nedkov et al., 1985, Biol. Chem. Hoppe-Seyler 366:421-430; Svendsen et al., 1986, FEBS 25 Lett 196:228-232), and two fungal subtilisins, subtilisin thermitase from Thermoactinomyces vulgaris (Meloun et al., 1985, FEBS. Lett. 183:195-200) and proteinase K from Tritirachium album (Jany and Mayer, 1985, Biol. Chem. Hoppe-Seyler 366:584-492). 30

Subtilisins are well-characterized physically and chemically. In addition to knowledge of the primary structure (amino acid sequence) of these enzymes, over 50

-5-

high resolution X-ray structures of subtilisin have been determined which delineate the binding of substrate, transition state, products, three different protease inhibitors, and define the structural consequences for natural variation (Kraut, 1977, Ann. Rev. Biochem. 46:331-358). Random and site-directed mutations of the subtilisin gene have both arisen from knowledge of the physical and chemical properties of the enzyme and contributed information relating to subtilisin's catalytic activity, substrate specificity, tertiary structure, etc. (Wells et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84: 1219-1223; Wells et al., 1986, Phil. Trans. R. Soc. Lond. A. 317:415-423; Hwang and Warshel, 1987, Biochem. 26:2669-2673; Rao et al., 1987, Nature 328:551-554).

15

### 2.3. INDUSTRIAL APPLICATIONS OF SUBTILISINS

Subtilisins have found much utility in industry, particularly detergent formulations, as they are useful for removing proteinaceous stains. To be effective, however, these enzymes must not only possess activity under washing conditions, but must also be compatible with other detergent components during storage. For example, subtilisin may be used in combination with amylases, which are active against starches; cellulases which will digest cellulosic materials; lipases, which are active against fats; peptidases, which are active on peptides, and ureases, which are effective against urine stains. Not only must the formulation protect other enzymes from digestion by subtilisin, but subtilisin must be stable with respect to the oxidizing power, calcium binding properties, detergency and high pH of nonenzymatic detergent components. The ability of the enzyme to remain stable in their presence is often referred to as its washing ability or washability.

35

-6-

### 3. SUMMARY OF THE INVENTION

The present invention relates to mutations of the subtilisin gene, some of which result in changes in the chemical characteristics of subtilisin enzyme. Mutations are created at specific nucleic acids of the subtilisin gene, and, in various specific embodiments, the mutant enzymes possess altered chemical properties including, but not limited to, increased stability to oxidation, augmented proteolytic ability, and improved washability.

The present invention also relates, but is not limited to the amino acid and DNA sequences for two proteases derived from Bacillus lentus variants, subtilisin 147 and subtilisin 309, as well as mutations of these genes and the corresponding mutant enzymes.

Site-directed mutation can efficiently produce mutant subtilisin enzymes which can be tailored to suit a multitude of industrial applications particularly in the areas of detergent and food technology. The present invention relates, in part, but is not limited to, mutants of the subtilisin 309 gene which exhibit improved stability to oxidation, augmented protease activity, and/or improved washability.

#### 3.1. ABBREVIATIONS

25	A	=	Ala	=	Alanine
	V	=	Val	=	Valine
	L	=	Leu	=	Leucine
	I	=	Ile	=	Isoleucine
	P	=	Pro	=	Proline
	F	=	Phe	=	Phenylalanine
30	W	=	Trp	=	Tryptophan
	M	=	Met	=	Methionine
	G	=	Gly	=	Glycine



-7-

	S	=	Ser	=	Serine
	T	=	Thr	=	Threonine
	C	=	Cys	=	Cysteine
	Y	=	Tyr	=	Tyrosine
5	N	=	Asn	=	Asparagine
	Q	=	Gln	=	Glutamine
	D	=	Asp	=	Aspartic Acid
	E	=	Glu	=	Glutamic Acid
	K	=	Lys	=	Lysine
10	R	=	Arg	=	Arginine
	H	=	His	=	Histidine

#### 4. DESCRIPTION OF THE FIGURES

Figure 1 illustrates the insertion of a subset of fragments, ranging from 1.5kb to 6.5kb in length, generated by partial digestion of Bacillus lentus strain 309 DNA with Sau 3A restriction endonuclease, into Bam HI cut plasmid pSX50. The two resulting plasmids, pSX86 and pSX88, containing the subtilisin 309 gene in opposite orientations, are also shown.

Figure 2 illustrates the insertion of Bacillus lentus strain 147 DNA fragments into plasmid pSX56. Partial digestion of strain 147 DNA was performed using Sau 3A restriction endonuclease. Fragments ranging in size from 1.5 to 6.5 kb were then ligated into Bam HI cleaved plasmid pSX56. The product, pSX94, contains the subtilisin 147 gene.

Figure 3 illustrates gapped duplex mutagenesis, using the method of Morinaga et al., (1984, Biotechnology 2: 636-639). It features two plasmids, pSX93 and pSX119, both derived from pUC13. pSX93 contains an XbaI-HindIII fragment of the subtilisin 309 gene, and pSX119 contains the remainder of the subtilisin 309 gene in an EcoRI-XbaI

-8-

fragment. In (A), plasmid pSX93 is cleaved with XbaI and ClaI, and the gapped molecules are mixed with pSX93 cut with ScaI, denatured, and allowed to reanneal so as to generate plasmids with a region of single-stranded DNA extending within the subtilisin 309 coding sequence. A synthetic oligonucleotide, homologous to the subtilisin 309 gene but containing a mutation, is allowed to anneal to the single stranded gap, which is then filled in using the Klenow fragment of DNA polymerase I and T4 DNA ligase. Upon replication of the plasmid, double-stranded mutants of the subtilisin 309 gene are generated. The same procedure is performed in (B), using plasmid pSX119 and EcoRI and XbaI enzymes, to create mutations in the corresponding region of the subtilisin 309 gene.

Figure 4 illustrates plasmid pSX92, which is a derivative of plasmid pSX62, bearing the subtilisin 309 gene. Mutated fragments (i.e., XbaI - ClaI, XbaI-HindIII, or EcoRI-XbaI), excised from mutation plasmid pSX93 or pSX119 (see Figure 3) using the appropriate restriction endonucleases, were inserted into plasmid pSX92 for expression in B. subtilis strain DN 497.

Figure 5 illustrates plasmid pSX143, which contains truncated forms of both subtilisin 309 and subtilisin 147 genes. In vivo recombination between homologous regions of the two genes can result in active protease.

##### 5. DETAILED DESCRIPTION OF THE INVENTION

The invention relates to mutations of the subtilisin gene, some of which result in changes in the chemical characteristics of subtilisin enzyme. Mutations at specific nucleic acids may be generated, and thus, forms of subtilisin can be designed so as to meet the needs of industrial application.

-9-

The invention is based, in part, upon the discovery that mutations of specific nucleic acids in the subtilisin gene can result in enzymes with altered properties. In various embodiments, enzymes with improved stability to oxidation, augmented protease activity, or improved washing ability can be generated.

For purposes of clarity in description, and not by way of limitation, the invention will be described in four parts: (a) the chemical structure of known subtilisins and subtilisin 147 and 309; (b) methods for producing mutations in the subtilisin gene; (c) expression of mutants of subtilisin and (d) screening of subtilisin mutants for desirable chemical properties.

#### 5.1. CHEMICAL STRUCTURES OF KNOWN SUBTILISINS AND SUBTILISIN 147 AND 309

Sequence analysis of subtilisin from various sources can reveal the functional significance of the primary amino acid sequence, and can direct the creation of new mutants with deliberately modified functions. Comparing the amino acid sequence of different forms of subtilisin, while contrasting their physical, or chemical properties, may reveal specific target regions which are likely to produce useful mutant enzymes.

The amino acid sequences of at least eight subtilisins are known. These include six subtilisins from Bacillus strains, namely, subtilisin 168, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin amylosacchariticus and mesenticopeptidase (Kurihara et al., 1972, J. Biol. Chem. 247:5629-5631; Stahl and Ferrari, 1984, J. Bacteriol. 158:411-418; Vasantha et al., 1984, J. Bacteriol. 159:811-819; Jacobs et al., 1985, Nucl. Acids Res. 13:8913-8926; Nedkov et al., 1985, Biol. Chem. Hoppe-Seyler 366:421-430;

-10-

Svendsen et al., 1986, FEBS Lett. 196:228-232), and two fungal subtilisins, subtilisin thermitase from *Thermoactinomyces vulgaris* (Meloun et al., 1985, FEBS Lett. 183:195-200), and proteinase K from *Tritirachium album* 5 limber (Jany and Mayer, 1985, Biol. Chem. Hoppe-Seyler 366:485-492).

In connection with this invention the amino acid and DNA sequences for two further serine proteases are revealed. These proteases were derived from two *Bacillus lentus* 10 variants, 147 and 309, which have been deposited with NCIB and designated the accession Nos. NCIB 10147 and NCIB 10309, respectively. For convenience the proteases produced by these strains are designated subtilisin 147 and subtilisin 309, respectively, and the genes encoding these proteins are 15 referred to as the subtilisin 147 and 309 genes.

As used in this invention the term "subtilisin material" refers to a proteinaceous material which contains a subtilisin as its active ingredient. As used herein, and under the definition of subtilisin material, any serine 20 protease is a subtilisin which has at least 30%, preferably 50%, and more preferably 80% amino acid sequence homology with the sequences referenced above for subtilisin 147, subtilisin 309, subtilisin 168, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin amylosacchariticus, mesenticopeptidase, thermitase, proteinase K and 25 thermomycolase. These serine proteases are also described herein as "homologous serine proteases".

Table I compares the deduced amino acid sequences of subtilisin 309, subtilisin 147, subtilisin BPN', subtilisin 30 Carlsberg and subtilisin 168 (Spizizen, et al., 1958, Proc. Natl. Acad. Sci. U.S.A. 44:1072-1078). Table II presents the nucleic acid sequence of the subtilisin 309 gene, and Table III presents the nucleic acid sequence of the

-11-

subtilisin 147 gene. The sequences of subtilisin 309 or 147, or their functional equivalents, can be used in accordance with the invention. For example, the sequences of subtilisin 309 or 147 depicted in Tables I, II or III can be altered by substitutions, additions or deletions that provide for functionally equivalent molecules. For example, due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as depicted in Table I may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of the subtilisin 309 or 147 sequences depicted in Tables II or III which are altered by the substitution of different codons that encode the same or a functionally equivalent amino acid residues within the sequence, thus producing a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic and glutamic acid.

Closeness of relation can be measured by comparison of amino-acid sequences. There are many methods of aligning protein sequences, but the differences are only manifest when the degree of relatedness is quite small. The methods described in Atlas of Protein Sequence and Structure,

-12-

Margaret O. Dayhoff editor, vol. 5, supplement 2, 1976, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D.C., p. 3 ff., entitled SEARCH and ALIGN, define relatedness. As is well known in the art, related proteins can differ in number of amino acids as well as identity of each amino acid along the chain. That is, there can be deletions or insertions when two structures are aligned for maximum identity. For example, subtilisin Carlsberg has only 274 amino acids while subtilisin BPN' has 275 amino acids. Aligning the two sequences shows that Carlsberg has no residue corresponding to Asn56 of subtilisin BPN'. Thus the amino acid sequence of Carlsberg would appear very different from BPN' unless a gap is recorded at location 56. Therefore, one can predict with a high degree of confidence that substituting Ser for Asn at location 218 of subtilisin Carlsberg will increase thermal stability provided that the residues in Carlsberg are numbered by homology to BPN'.

According to the invention, the sequences determined for subtilisins 309 and 147 can be compared with sequences of known subtilisins (see Table I) or newly discovered subtilisins in order to deduce sites for desirable mutations. To do this, the closeness of relation of the subtilisins being compared must be determined.

Experiments to determine the relationship between the primary structure of subtilisin and its physical properties have revealed the significance of the methionine-222 residue as well as the amino acids functional in the active site, namely, aspartic acid-32, histidine-64, and serine-221. Asparagine-155 and Serine-221 are within the oxyanion binding site. Mutations at these positions are likely to diminish proteolytic activity. According to the present invention, the amino acid sequences of subtilisins 309 and

-13-

147 were compared with one another and with the sequences of other subtilisins (see Table II). Residues that varied between subtilisin 309 or 147 and other subtilisins were identified. For example, at residue 153, subtilisin 309 contains a serine residue, whereas subtilisin 147, BPN', Carlsberg and 168 contain an alanine residue. Therefore, if the serine 153 residue of subtilisin 309 were changed to an alanine residue, the physical properties of subtilisin 309 might be altered in a desired direction. Likewise, subtilisin 147 contains a serine residue at position 218, whereas the other subtilisins expressed an asparagine residue. Because subtilisin 147 has improved thermal stability relative to the other subtilisins, mutating the asparagine 218 of subtilisin 309 to a serine residue might improve the thermal stability of subtilisin 309. As another example, it was reasoned that, since Thr 71 is close to the active site, the introduction of a negatively charged amino acid, such as aspartic acid, might suppress oxidative attack by electrostatic repulsion. The sites that are most likely to be relevant to the physical properties of subtilisin are those in which there is conservation of amino residues between most subtilisins, for example Asp-153 and Asn-218 discussed above, and also Trp-6, Arg-170, Pro-168, His-67, Met-175, Gly-219, Arg-275. By mutating the nucleic acid sequence such that a amino acid which differs from other subtilisins is substituted with an amino acid that conforms, a more stable form of subtilisin may result.

Wells et al. (1987, Proc. Natl. Acad. Sci. U.S.A. 84:1219-1223) have used comparison of amino acid sequences and site-directed mutation to engineer subtilisin substrate specificity. The catalytic activities of various subtilisins can differ markedly against selected substrates. Wells has shown that only three amino acid substitutions can

-14-

cause B. amyloliquefaciens subtilisin substrate specificity to approach that of B. lichenformis subtilisin, enzymes that differ by factors of 10-50 in catalytic efficiency in their native state. Comparison analysis between subtilisin 147 and 309 and other subtilisins has indicated that mutation of the following sites may alter the physical or chemical properties of subtilisin: 6, 9, 11-12, 19, 25, 36-38, 53-59, 67, 71, 89, 104, 111, 115, 120, 121-122, 124, 128, 131, 140, 153-166, 168, 169-170, 172, 175, 180, 182, 186, 187, 191, 194, 195, 199, 218, 219, 222, 226, 234-238, 241, 260-262, 265, 268, or 275. Deletions occur at the following sites in subtilisins 147 and/or 309; insertion of appropriate amino acid residues into these sights might enhance the stability of the parent enzymes: 1, 36, 56, 159, 164-166. According to the method illustrated by these examples, which are not limiting, a number of potential mutation sites become apparent.

-----

Table I

COMPARISON OF AMINO ACID SEQUENCE FOR VARIOUS PROTEASES

	10	20	30
a)	A-Q-S-V-P-W-G-I-S-R-V-Q-A-P-A-A-H-N-R-G-L-T-G-S-G-V-K-V-A-V-		
b)	°-Q-T-V-P-W-G-I-S-F-I-N-T-Q-Q-A-H-N-R-G-I-F-G-N-G-A-R-V-A-V-		
c)	A-Q-S-V-P-Y-G-V-S-Q-I-K-A-P-A-L-H-S-Q-G-Y-T-G-S-N-V-K-V-A-V-		
d)	A-Q-T-V-P-Y-G-I-P-L-I-K-A-D-K-V-Q-A-Q-G-F-K-G-A-N-V-K-V-A-V-		
e)	A-Q-S-V-P-Y-G-I-S-Q-I-K-A-P-A-L-H-S-Q-G-Y-T-G-S-N-V-K-V-A-V-		



-15-

- |    | 40  | 50  | 60  |
|----|---|-----|-----|
|    | a) L-D-T-G-I-°-S-T-H-P-D-L-N-I-R-G-G-A-S-F-V-P-G-E-P-°-S-T-Q-D- |     |     |
|    | b) L-D-T-G-I-°-A-T-H-P-D-L-R-I-A-G-G-A-S-F-I-S-S-E-P-°-S-Y-H-D- |     |     |
| 5  | c) I-D-S-G-I-D-S-S-H-P-D-L-K-V-A-G-G-A-S-M-V-P-S-E-T-N-P-F-Q-D- |     |     |
|    | d) L-D-T-G-I-Q-A-S-H-P-D-L-N-V-V-G-G-A-S-F-V-A-G-E-A-°-Y-N-T-D- |     |     |
|    | e) L-D-S-G-I-D-S-S-H-P-D-L-N-V-R-G-G-A-S-F-V-A-S-E-T-N-P-Y-Q-D- |     |     |
|    |   |     |     |
| 10 | 70  | 80  | 90  |
|    | a) G-N-G-H-G-T-H-V-A-G-T-I-A-A-L-N-N-S-I-G-V-L-G-V-A-P-S-A-E-L- |     |     |
|    | b) N-N-G-H-G-T-H-V-A-G-T-I-A-A-L-N-N-S-I-G-V-L-G-V-A-P-S-A-D-L- |     |     |
|    | c) N-N-S-H-G-T-H-V-A-G-T-V-A-A-L-N-N-S-I-G-V-L-G-V-A-P-S-A-S-L- |     |     |
| 15 | d) G-N-G-H-G-T-H-V-A-G-T-V-A-A-L-D-N-T-T-G-V-L-G-V-A-P-S-V-S-L- |     |     |
|    | e) G-S-S-H-G-T-H-V-A-G-T-I-A-A-L-N-N-S-I-G-V-L-G-V-S-P-S-A-S-L- |     |     |
|    |   |     |     |
|    | 100   | 110 | 120 |
| 20 | a) Y-A-V-K-V-L-G-A-S-G-S-G-S-V-S-S-I-A-Q-G-L-E-W-A-G-N-N-G-M-H- |     |     |
|    | b) Y-A-V-K-V-L-D-R-N-G-S-G-S-L-A-S-V-A-Q-G-I-E-W-A-I-N-N-N-M-H- |     |     |
|    | c) Y-A-V-K-V-L-G-A-D-G-S-G-Q-Y-S-W-I-I-N-G-I-E-W-A-I-A-N-N-M-D- |     |     |
|    | d) Y-A-V-K-V-L-N-S-S-G-S-G-T-Y-S-G-I-V-S-G-I-E-W-A-T-T-N-G-M-D- |     |     |
| 25 | e) Y-A-V-K-V-L-D-S-T-G-S-G-Q-Y-S-W-I-I-N-G-I-E-W-A-I-S-N-N-M-D- |     |     |
|    |   |     |     |
|    | 130   | 140 | 150 |
|    | a) V-A-N-L-S-L-G-S-P-S-P-S-A-T-L-E-Q-A-V-N-S-A-T-S-R-G-V-L-V-V- |     |     |
| 30 | b) I-I-N-M-S-L-G-S-T-S-G-S-S-T-L-E-L-A-V-N-R-A-N-N-A-G-I-L-L-V- |     |     |
|    | c) V-I-N-M-S-L-G-G-P-S-P-S-A-A-L-K-A-A-V-D-K-A-V-A-S-G-V-V-V-V- |     |     |
|    | d) V-I-N-M-S-L-G-G-P-S-G-S-T-A-M-K-Q-A-V-D-N-A-Y-A-R-G-V-V-V-V- |     |     |
|    | e) V-I-N-M-S-L-G-G-P-T-G-S-A-A-L-K-T-V-V-D-K-A-V-S-S-G-I-L-V-A- |     |     |

-16-

- |    |    |  |     |     |
|----|----|--|-----|-----|
|    |    | 160  | 170 | 180 |
|    | a) | A-A-S-G-N-S-G-A-°-G-S-I-S-°-°-°-Y-P-A-R-Y-A-N-A-M-A-V-G-A-T- |     |     |
|    | b) | G-A-A-G-N-T-G-R-°-Q-G-V-N-°-°-°-Y-P-A-R-Y-S-G-V-M-A-V-A-A-V- |     |     |
| 5  | c) | A-A-A-G-N-E-G-T-S-G-S-S-S-T-V-G-Y-P-G-X-Y-P-S-V-I-A-V-G-A-V- |     |     |
|    | d) | A-A-A-G-N-S-G-S-S-G-N-T-N-T-I-G-Y-P-A-K-Y-D-S-V-I-A-V-G-A-V- |     |     |
|    | e) | A-A-A-G-N-E-G-S-S-G-S-S-S-T-V-G-Y-P-A-K-Y-P-S-T-I-A-V-G-A-V- |     |     |
| 10 |    | 190  | 200 | 210 |
|    | a) | D-Q-N-N-N-R-A-S-F-S-Q-Y-G-A-G-L-D-I-V-A-P-G-V-N-V-Q-S-T-Y-P- |     |     |
|    | b) | D-Q-N-G-Q-P-P-S-F-S-T-Y-G-P-E-I-E-I-S-A-P-G-V-N-V-N-S-T-Y-T- |     |     |
|    | c) | D-S-S-N-Q-R-A-S-F-S-S-V-G-P-E-L-D-V-M-A-P-G-V-S-I-Q-S-T-L-P- |     |     |
| 15 | d) | D-S-N-S-N-R-A-S-F-S-S-V-G-A-E-L-E-V-M-A-P-G-A-G-V-Y-S-T-Y-P- |     |     |
|    | e) | N-S-S-N-Q-R-A-S-F-S-S-A-G-S-E-L-D-V-M-A-P-G-V-S-I-Q-S-T-L-P- |     |     |
| 20 |    | 220  | 230 | 240 |
|    | a) | G-S-T-Y-A-S-L-N-G-T-S-M-A-T-P-H-V-A-G-A-A-A-L-V-K-Q-K-N-P-S- |     |     |
|    | b) | G-N-R-Y-V-S-L-S-G-T-S-M-A-T-P-H-V-A-G-V-A-A-L-V-K-S-R-Y-P-S- |     |     |
|    | c) | G-N-K-Y-G-A-Y-N-G-T-S-M-A-S-P-H-V-A-G-A-A-A-L-I-L-S-K-H-P-N- |     |     |
|    | d) | T-S-T-Y-A-T-L-N-G-T-S-M-A-S-P-H-V-A-G-A-A-A-L-I-L-S-K-H-P-N- |     |     |
| 25 | e) | G-G-T-Y-G-A-Y-N-G-T-S-M-A-T-P-H-V-A-G-A-A-A-L-I-L-S-K-H-P-T- |     |     |

30

35

-17-

250

260

270

- 5 a) W-S-N-V-Q-I-R-N-H-L-K-N-T-A-T-S-L-G-S-T-N-L-Y-G-S-G-L-V-N-A-  
 b) Y-T-N-N-Q-I-R-Q-R-I-N-Q-T-A-T-Y-L-G-S-P-S-L-Y-G-N-G-L-V-H-A-  
 c) W-T-N-T-Q-V-R-S-S-L-E-N-T-T-T-K-L-G-D-S-F-Y-Y-G-K-G-L-I-N-V-  
 d) L-S-A-S-Q-V-R-N-R-L-S-S-T-A-T-Y-L-G-S-S-F-Y-Y-G-K-G-L-I-N-V-  
 e) W-T-N-A-Q-V-R-D-R-L-E-S-T-A-T-Y-L-G-N-S-F-Y-Y-G-K-G-L-I-N-V-

- 10 a) E-A-A-T-R  
 b) G-R-A-T-Q  
 c) Q-A-A-A-Q  
 d) E-A-A-A-Q  
 e) Q-A-A-A-Q

15

- a = subtilisin 309  
 b = subtilisin 147  
 c = subtilisin BPN'  
 20 d = subtilisin Carlsberg  
 e = subtilisin 168  
 e = assigned deletion

25 5.2. METHODS FOR PRODUCING MUTATIONS  
IN SUBTILISIN GENES

Many methods for introducing mutations into genes are well known in the art. After a brief discussion of cloning subtilisin genes, methods for generating mutations in both random sites, and specific sites, within the subtilisin gene  
 30 will be discussed.

35

-18-

#### 5.2.1. CLONING A SUBTILISIN GENE

The gene encoding subtilisin may be cloned from any Gram-positive bacteria or fungus by various methods, well known in the art. First a genomic, and/or cDNA library of DNA must be constructed using chromosomal DNA or messenger RNA from the organism that produces the subtilisin to be studied. Then, if the amino-acid sequence of the subtilisin is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify subtilisin-encoding clones from a genomic library of bacterial DNA, or from a fungal cDNA library. Alternatively, a labelled oligonucleotide probe containing sequences homologous to subtilisin from another strain of bacteria or fungus could be used as a probe to identify subtilisin-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying subtilisin-producing clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming protease-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for subtilisin, such as skim milk. Those bacteria containing subtilisin-bearing plasmid will produce colonies surrounded by a halo of clear agar, due to digestion of the skim milk by excreted subtilisin.

#### 5.2.2. GENERATION OF RANDOM MUTATIONS IN THE SUBTILISIN GENE

Once the subtilisin gene has been cloned into a suitable vector, such as a plasmid, several methods can be used to introduce random mutations into the gene.

One method would be to incorporate the cloned subtilisin gene, as part of a retrievable vector, into a mutator strain of Eschericia coli.

-19-

Another method would involve generating a single stranded form of the subtilisin gene, and then annealing the fragment of DNA containing the subtilisin gene with another DNA fragment such that a portion of the subtilisin gene remained single stranded. This discrete, single stranded region could then be exposed to any of a number of mutagenizing agents, including, but not limited to, sodium bisulfite, hydroxylamine, nitrous acid, formic acid, or hydralazine. A specific example of this method for generating random mutations is described by Shortle and Nathans (1978, Proc. Natl. Acad. Sci. U.S.A., 75:2170-2174). According to the Shortle and Nathans method, the plasmid bearing the subtilisin gene would be nicked by a restriction enzyme that cleaves within the gene. This nick would be widened into a gap using the exonuclease action of DNA polymerase I. The resulting single-stranded gap could then be mutagenized using any one of the above mentioned mutagenizing agents.

Alternatively, the subtilisin gene from a Bacillus species including the natural promoter and other control sequences could be cloned into a plasmid vector containing replicons for both E. coli and B. subtilis, a selectable phenotypic marker and the M13 origin of replication for production of single-stranded plasmid DNA upon superinfection with helper phage IR1. Single-stranded plasmid DNA containing the cloned subtilisin gene is isolated and annealed with a DNA fragment containing vector sequences but not the coding region of subtilisin, resulting in a gapped duplex molecule. Mutations are introduced into the subtilisin gene either with sodium bisulfite, nitrous acid or formic acid or by replication in a mutator strain of E. coli as described above. Since sodium bisulfite reacts exclusively with cytosine in a single-stranded DNA, the

-20-

mutations created with this mutagen are restricted only to the coding regions. Reaction time and bisulfite concentration are varied in different experiments such that from one to five mutations are created per subtilisin gene on average. Incubation of 10  $\mu$ g of gapped duplex DNA in 4 M Na-bisulfite, pH. 6.0, for 9 minutes at 37°C in a reaction volume of 400  $\mu$ l, deaminates about 1% of cytosines in the single-stranded region. The coding region of mature subtilisin contains about 200 cytosines, depending on the DNA strand. Advantageously, the reaction time is varied from about 4 minutes (to produce a mutation frequency of about one in 200) to about 20 minutes (about 5 in 200).

After mutagenesis the gapped molecules are treated in vitro with DNA polymerase I (Klenow fragment) to make fully double-stranded molecules and to fix the mutations. Competent E. coli are then transformed with the mutagenized DNA to produce an amplified library of mutant subtilisins. Amplified mutant libraries can also be made by growing the plasmid DNA in a Mut D strain of E. coli which increases the range of mutations due to its error prone DNA polymerase.

The mutagens nitrous acid and formic acid may also be used to produce mutant libraries. Because these chemicals are not as specific for single-stranded DNA as sodium bisulfite, the mutagenesis reactions are performed according to the following procedure. The coding portion of the subtilisin gene is cloned in M13 phage by standard methods and single stranded phage DNA prepared. The single-stranded DNA is then reacted with 1 M nitrous acid pH. 4.3 for 15-60 minutes at 23°C or 2.4 M formic acid for 1-5 minutes at 23°C. These ranges of reaction times produce a mutation frequency of from 1 in 1000 to 5 in 1000. After mutagenesis, a universal primer is annealed to the M13 DNA and duplex DNA is synthesized using the mutagenized single-

-21-

stranded DNA as a template so that the coding portion of the subtilisin gene becomes fully double-stranded. At this point the coding region can be cut out of the M13 vector with restriction enzymes and ligated into an unmutagenized expression vector so that mutations occur only in the restriction fragment. (Myers et al., Science 229:242-257 (1985)).

By yet another method, mutations can be generated by allowing two dissimilar forms of subtilisin to undergo recombination in vivo. According to this method, homologous regions within the two genes lead to a cross-over of corresponding regions resulting in the exchange of genetic information. The generation of hybrid amylase molecules according to this technique is fully described in U.S. patent application serial number 67,992, filed on June 29, 1987, which is incorporated by reference in its entirety herein. An example of a plasmid which can generate hybrid forms of subtilisin is depicted in Figure 5. Both the subtilisin 309 and 147 genes, incorporated into plasmid pSX143, are truncated, and therefore cannot themselves lead to subtilisin expression. However, if recombination occurs between the two genes so as to correct the defect produced by truncation, i.e., the N terminal region of the subtilisin 309 gene becomes linked to the C terminal region of the subtilisin 147 gene, then active, mutant subtilisin can be produced. If pSX143 is incorporated into a protease-negative strain of bacteria, and then bacteria who develop a protease positive phenotype are selected, then various mutants, subtilisin 309/147 chimeras, can be identified.

30

35

-22-

### 5.2.3. GENERATION OF SITE DIRECTED MUTATIONS IN THE SUBTILISIN GENE

Once the subtilisin gene has been cloned, and desirable sites for mutation identified, these mutations can be introduced using synthetic oligo nucleotides. These  
5 oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a preferred method, a single stranded gap of DNA, bridging the subtilisin gene, is created in a vector bearing the subtilisin gene. Then the  
10 synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in by DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in  
15 Morinaga et al., (1984, Biotechnology 2:646-639). According to Morinaga et al., a fragment within the gene is removed using restriction endonuclease. The vector/gene, now containing a gap, is then denatured and hybridized to vector/gene which, instead of containing a gap, has been  
20 cleaved with another restriction endonuclease at a site outside the area involved in the gap. A single-stranded region of the gene is then available for hybridization with mutated oligonucleotides, the remaining gap is filled in by the Klenow fragment of DNA polymerase I, the insertions are  
25 ligated with T4 DNA ligase, and, after one cycle of replication, a double-stranded plasmid bearing the desired mutation is produced. The Morinaga method obviates the additional manipulation of construction new restriction sites, and therefore facilitates the generation of mutations  
30 at multiple sites. U.S. Patent number 4,760,025, by Estelle et al., issued July 26, 1988, is able to introduce oligonucleotides bearing multiple mutations by performing



-23-

minor alterations of the cassette, however, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

5

### 5.3. EXPRESSION OF SUBTILISIN MUTANTS

According to the invention, a mutated subtilisin gene produced by methods described above, or any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector. An expression vector generally falls under the definition of a cloning vector, since an expression vector usually includes the components of a typical cloning vector, namely, an element that permits autonomous replication of the vector in a microorganism independent of the genome of the microorganism, and one or more phenotypic markers for selection purposes. An expression vector includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene. To permit the secretion of the expressed protein, nucleotides encoding a "signal sequence" may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a target gene to be treated according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can support the transcription of the mutant subtilisin gene, include but are not limited to the prokaryotic  $\beta$ -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94.

-24-

According to one embodiment B. subtilis is transformed by an expression vector carrying the mutated DNA. If expression is to take place in a secreting microorganism such as B. subtilis a signal sequence may follow the translation initiation signal and precede the DNA sequence of interest. The signal sequence acts to transport the expression product to the cell wall where it is cleaved from the product upon secretion. The term "control sequences" as defined above is intended to include a signal sequence, when it is present.

#### 5.4. SCREENING OF MUTANT SUBTILISINS

For screening mutants, transformed B. subtilis can be cultivated in the presence of a filter material (such as nitrocellulose) to which the secreted expression product (e.g. enzyme) binds. In order to screen for an expression product having a desired characteristic, filter bound expression product is subjected to conditions which distinguish expression product of interest from wild-type expression product. For example, the filter-bound expression product can be subjected to conditions which would inactivate a wild-type product. Preserved enzyme activity following adverse treatment suggests that the mutation confers enhanced stability on the enzyme, and is therefore a useful mutation.

In one embodiment of the invention, screening for stable variants is accomplished using a protease deficient B. subtilis strain transformed with the variant plasmid and plated out as follows: a nitrocellulose filter is placed on a nutrient base in a petri dish, and a cellulose acetate filter is placed on top of the nitrocellulose. Colonies are grown on the cellulose acetate, and protease from individual colonies is secreted through the cellulose acetate onto the

-25-

nitrocellulose filter where it is stably bound. Protease from hundreds of colonies is bound to a single filter allowing subsequent screening of thousands of different variants by processing multiple filters.

5 To identify colonies producing subtilisin of enhanced thermal stability, the filters can be incubated in buffer solutions at temperatures which would inactivate substantially all wild-type activity. Variants of enhanced stability or activity retain activity after this step. The  
10 suitably treated filter then is soaked in a solution containing Tosyl-L-Arg methyl ester (TAME) Benzoyl-Arg-ethyl-ester (BAEE), Acetyl-Tyr-ethyl-ester (ATEE) (Sigma) or similar compounds. Because TAME, BAEE, and ATEE are substrates for the proteases they are cleaved in zones on  
15 the filter containing variant subtilisins which remain active after treatment. As cleavage occurs, protons are released in the reaction and cause phenol red to change in color from red to yellow in areas retaining protease activity.

20 This procedure can be used to screen for different classes of variants with only slight modifications. For example, the filters could be treated at high temperature, at high pH, with denaturants, oxidizing agents, or under other conditions which normally inactivate an enzyme such as  
25 a protease to find resistant variants. Variants with altered substrate specificity could be screened by replacing TAME, BAEE, or ATEE with other substrates which are normally not cleaved by wild-type subtilisin.

Once a variant of enhanced stability is identified  
30 by screening, the colony from which the variant is derived is isolated and the altered subtilisin is purified. Experiments can be performed on the purified enzyme to determine conditions of stability towards oxidation, thermal

-26-

inactivation, denaturation temperature, kinetic parameters as well as other physical measurements. The altered gene can also be sequenced to determine the amino acid changes responsible for the enhanced stability. Using this  
5 procedure, variants with increased washing abilities have been isolated.

6. EXAMPLE: SITE-SPECIFIC MUTATION OF THE SUBTILISIN GENE GENERATES MUTANTS WITH USEFUL CHEMICAL CHARACTERISTICS

6.1. MATERIALS AND METHODS

6.1.1. BACTERIAL STRAINS

B. subtilis 309 and 147 are variants of Bacillus  
15 lentus, deposited with the NCIB and accorded the accession numbers NCIB 10147 and NCIB 10309, and described in U.S. Patent No. 3,723,250, issued March 27, 1973, and incorporated in its entirety by reference herein. B. subtilis DN 497 is described in U. S. Serial No. 039,298,  
20 also incorporated by reference herein, and is an  $aro^+$  transformant of RUB 200 with chromosomal DNA from SL 438, a sporulation and protease deficient strain obtained from Dr. Kim Hardy of Biogen. E. coli MC 1000  $r^-m^+$  (Casa-daban, M.J. and Cohen, S.N. (1980), J. Mol. Biol. 138:179-207, was made  
25  $r^-m^+$  by conventional methods and is also described in U. S. Serial No. 039,298.

6.1.2. PLASMIDS

pSX50 (described in U.S. patent application serial  
30 No. 039,298 filed April 17, 1987, and incorporated by reference herein) is a derivative of plasmid pDN 1050,

-27-

comprising the promoter-operator  $P_{1O_1}$ , the B. pumilus xyn B gene and the B. subtilis xyl R gene.

pSX65 (described in U.S. patent application serial No. 039,298, supra) is a derivative of plasmid pDN 1050, comprising the promoter-operator  $P_{2O_2}$ , the B. pumilus xyn B gene, and the B. subtilis xyl R gene.

pSX93, shown in Figure 3a, is pUC13 (Vieira and Messing, 1982, Gene 10:259-268) comprising a 0.7kb XbaI-Hind III fragment of the subtilisin 309 gene including the terminator inserted in a polylinker sequence.

pSX119 is pUC13 harboring an EcoRI-XbaI fragment of the subtilisin 309 gene inserted into the polylinker.

pSX62 (described in U.S. patent application serial No. 039,298, supra) is a derivative of pSX52 (ibid), which comprises a fusion gene between the calf prochymosin gene and the B. pumilus xyn B gene inserted into pSX50 (supra). pSX62 was generated by inserting the E. coli rrn B terminator into pSX52 behind the prochymosin gene.

pSX92 was produced by cloning the subtilisin 309 into plasmid pSX62 (supra) cut at Cla I and Hind III and filled prior to the insertion of the fragments DraI-NheI and NheI-Hind III from the cloned subtilisin 309 gene.

### 6.1.3. PURIFICATION OF SUBTILISINS

The procedure relates to a typical purification of a 10 liter scale fermentation of the Subtilisin 147 enzyme, the Subtilisin 309 enzyme or mutants thereof.

Approximately 8 liters of fermentation broth were centrifuged at 5000 rpm for 35 minutes in 1 liter beakers. The supernatants were adjusted to pH 6.5 using 10% acetic acid and filtered on Seitz Supra S100 filter plates.

The filtrates were concentrated to approximately 400 ml using an Amicon CH2A UF unit equipped with an Amicon

-28-

51Y10 UF cartridge. The UF concentrate was centrifuged and filtered prior to adsorption on a Bacitracin affinity column at pH 7. The protease was eluted from the Bacitracin column using 25% 2-propanol and 1 M sodium chloride in a buffer solution with 0.01 dimethylglutaric acid, 0.1 M boric acid and 0.002 M calcium chloride adjusted to pH 7.

The fractions with protease activity from the Bacitracin purification step were combined and applied to a 750 ml Sephadex G25 column (5 cm dia.) equilibrated with a buffer containing 0.01 dimethylglutaric acid, 0.2 M boric acid and 0.002 M calcium chloride adjusted to pH 6.5.

Fractions with proteolytic activity from the Sephadex G25 column were combined and applied to a 150 ml CM Sepharose CL 6B cation exchange column (5 cm dia.) equilibrated with a buffer containing 0.01 dimethylglutaric acid, 0.2 M boric acid and 0.002 M calcium chloride adjusted to pH 6.5.

The protease was eluted using a linear gradient of 0-0.1 M sodium chloride in 2 liters of the same buffer (0-0.2 M sodium chloride in case of sub 147).

In a final purification step protease containing fractions from the CM Sepharose column were combined and concentrated in an Amicon ultrafiltration cell equipped with a GR81P membrane (from the Danish Sugar Factories Inc.).

#### Subtilisin 309 and mutants

Met 222 to Ala  
Gly 195 to Glu  
Asn 218 to Ser  
Arg 170 to Tyr  
Gly 195 to Glu, Arg 170 to Tyr  
Gly 195 to Glu, Met 222 to Ala  
were purified by this procedure.

-29-

#### 6.1.4. OLIGONUCLEOTIDE SYNTHESIS

All mismatch primers were synthesized on an Applied Biosystems 380 A DNA synthesizer and purified by polyacrylamide gel electrophoresis (PAGE).

5

#### 6.1.5. DETERMINATION OF OXIDATION STABILITY

The purified enzyme is diluted to an enzyme content of approximately 0.1 mg/ml in 0.01 M dimethylglutaric acid pH 7 and in the same buffer with 0.01 M peracetic acid (pH 7).

10

Both sets of dilutions were heated to 50°C for 20 minutes. Proteolytic activity was measured in the dilutions before and after the heat treatment.

15

#### 6.1.6. ASSAY FOR PROTEOLYTIC ACTIVITY

##### OPA-Casein method

Proteolytic activity was determined using casein as the substrate. One Casein Protease Unit (CPU) is defined as the amount of enzyme liberating 1 millimole of primary amino groups (determined by comparison with a serine standard) per minute under standard conditions, i.e. incubation for 30 minutes at 25°C and pH 9.5.

20

A 2% (w/v) solution of casein (Hammarstein, supplied by Merck A.G., West Germany) was prepared with the Universal Buffer described by Britton and Robinson (Journ.Chem.Soc. 1931, p. 1451), adjusted to pH 9.5.

25

Two ml of substrate solution was preincubated in a water bath for 10 minutes at 25°C. 1 ml of enzyme solution containing about 0.2 - 0.3 CPU/ml of Britton-Robinson buffer (pH 9.5), was added. After 30 minutes of incubation at 25°C the reaction was terminated by the addition of a stopping agent (5 ml of a solution containing trichloroacetic acid (17.9 g), sodium acetate (29.9 g), and acetic acid (19.8 g),

30

35

-30-

filled up to 500 ml with deionized water). A blank was prepared in the same manner as the test solution, except that the stopping agent was added prior to the enzyme solution.

5           The reaction mixtures were kept for 20 minutes in the water bath, whereupon they were filtered through Whatman® 42 paper filters.

Primary amino groups were determined by their colour development with o-phthaldialdehyde (OPA).

10           Disodium tetraborate decahydrate (7.62 g) and sodium dodecylsulfate (2.0 g) was dissolved in 150 ml of water. OPA (160 mg) dissolved in 4 ml of methanol was then added together with 400 µl of beta-mercaptoethanol, whereafter the solution was made up to 200 ml with water.

15           To the OPA reagent (3 ml) was added 40 µl of the above-mentioned filtrates with mixing. The optical density (OD) at 340 nm was measured after about 5 minutes.

20           The OPA test was also performed with a serine standard containing 10 mg of serine in 100 ml of Britton-Robinson buffer (pH 9.5). The buffer was used as a blank.

The protease activity was calculated from the optical density measurements by means of the following formula:

$$25 \quad \text{CPU/ml of enzyme solution} = \frac{(\text{OD}_t - \text{OD}_b) \times C_{\text{ser}} \times Q}{(\text{OD}_{\text{ser}} - \text{OD}_B) \times \text{MW}_{\text{ser}} \times t_i}$$

$$\text{CPU/g of enzyme preparation} = \text{CPU/ml} : b$$

30           wherein  $\text{OD}_t$ ,  $\text{OD}_b$ ,  $\text{OD}_{\text{ser}}$  and  $\text{OD}_B$  is the optical density of the test solution, blank, serine standard, and buffer, respectively,  $C_{\text{ser}}$  the concentration of serine in mg/ml in



-31-

the standard,  $MW_{ser}$  the molecular weight of serine.  $Q$  is the dilution factor (in this instance equal to 8) for the enzyme solution, and  $t_1$  is the incubation time in minutes.

In the following Table V, results from the above  
5 assay are shown relative to the parent enzyme.

#### 6.1.7. ASSAY FOR WASHABILITY

Test cloths (7 cm x 7 cm, approximately 1 g) were produced by passing desized cotton (100% cotton, DS 71) cloth through the vessel in a Mathis Washing and Drying Unit  
10 type TH (Werner Mathis AG, Zurich, Switzerland) containing spinach juice (produced from fresh spinach) and then through the pressure roll of the machine in order to remove excess spinach juice.

15 Finally the cloth was dried in a strong air stream at room temperature, stored at room temperature for 3 weeks, and subsequently kept at -18°C prior to use.

The tests were performed in a Terg-O-tometer test washing machine (described in Jay C. Harris "Detergency Evaluation and Testing", Interscience Publishers Ltd., 1954,  
20 p. 60-61) isothermally for 10 minutes at 100 rpm. As detergent the following standard powder detergent was used:

	LAS, Nansa S 80	0.4 g/l
	AE, Berol 0 65	0.15 g/l
25	Soap	0.15 g/l
	STPP	1.75 g/l
	Sodium silicate	0.40 g/l
	CMC	0.05 g/l
	EDTA	0.01 g/l
30	$Na_2SO_4$	2.10 g/l
	Perborate	1.00 g/l
	TAED	0.10 g/l

-32-

TAED = N,N,N', N' -tetraacetyl-ethylene diamine; pH was adjusted with 4 N NaOH to 9.5. The water used was ca. 9°GH (German Hardness).

Tests were performed at enzyme concentrations of:  
5 0, 0.05 CPU/l and 0.1 CPU/l and two independent sets of tests were performed for each of the mutants.

Eight cloths were used for each testing using one beaker (800 ml) of detergent. Of the cloths, four were clean and four were stained with spinach juice. Subsequent  
10 to the washing the cloths were flushed in running water for 25 minutes in a bucket.

The cloths were then air dried overnight (protected against day light) and the remission, R, determined on a ElREPHO 2000 spectrophotometer from  
15 Datacolor S.A., Dietlikon, Switzerland at 460 nm.

As a measure of the washing ability differential remission,  $\Delta R$ , was used,  $\Delta R$  being equal to the remission after wash with enzyme added minus the remission after wash with no enzyme added.

20

#### 6.1.8. ASSAY FOR THERMOSTABILITY

The same procedure as above for washability was used for estimating the thermostability of the mutants produced, by performing the test at temperatures of 40°C and  
25 60°C, respectively.

### 6.2. RESULTS

#### 6.2.1. CLONING OF THE SUBTILISIN 309 AND 147 GENES

30

Chromosomal DNA from the "309" strain was isolated by treating a cell suspension with Lysozyme for 30 minutes

35

-33-

at 37°C, and then with SDS for 5 minutes at 60°C. Subsequently, the suspension was extracted with phenol-chloroform (50:50), precipitated with ethanol, and the precipitate redissolved in TE. This solution was treated with RNase for 1 hour at 37°C.

Approximately 30 µg of the chromosomal DNA was partially digested with restriction enzyme Sau 3A (New England Biolabs) and fragments from about 1.5 kb to about 6.5 kb were isolated on DEAE cellulose paper from a 1% agarose gel (the subtilisin gene in other species is approximately 1.2Kb in length).

As outlined in Figure 1 the fragments were annealed and ligated to BamHI cut plasmid pSX50 (described in US patent application No. 039,298 filed April 17, 1987, which is hereby included for reference). The plasmids were then transformed into competent B. subtilis DN 497.

The cells were then spread on LB agar plates with 10 mM phosphate pH 7, 6 µg/ml chloramphenicol, and 0.2% xylose to induce the xyn-promoter in the plasmid. The plates also contained 1% skim milk so the protease producing transformants could be detected by the clear halo where the skim milk had been degraded.

Protease expressing clones were produced at a frequency of  $10^{-4}$ . Two clones were found that harboured plasmids carrying the gene for subtilisin 309, pSX86 and pSX88. The gene was then sequenced using the method of Maxam and Gilbert. The deduced nucleotide sequence of subtilisin 309 is presented in Table II.

30

35

-34-

Table II

## THE SUBTILISIN 309 GENE

5	Signal				
	ATGAAGAAACCG	TTGGGGAAAATT	GTCGCAAGCACC	GCACTACTCATT	TCTGTTGCTTTT
	1	PRO			
	AGTTCATCGATC	GCATCGGCTGCT	GAAGAAGCAAAA	GAAAAATATTTA	ATTGGCTTTAAT
		82			
10	GAGCAGGAAGCT	GTCAGTGAGTTT	GTAGAACAAGTA	GAGGCAAATGAC	GAGGTCGCCATT
	CTCTCTGAGGAA	GAGGAAGTCGAA	ATTGAATTGCTT	CATGAATTTGAA	ACGATTCCTGTT
	TTATCCGTTGAG	TTAAGCCCAGAA	GATGTGGACGCG	CTTGAACTCGAT	CCAGCGATTTCT
			Mature		
	TATATTGAAGAG	GATGCAGAAGTA	ACGACAATGGCG	CAATCAGTGCCA	TGGGGAATTAGC
			334		
15	CGTGTGCAAGCC	CCAGCTGCCCCAT	AACCGTGGATTG	ACAGGTTCTGGT	GTAAAAGTTGCT
	GTCCTCGATACA	GGTATTTCCACT	CATCCAGACTTA	AATATTCGTGGT	GGCGCTAGCTTT
	GTACCAGGGGAA	CCATCCACTCAA	GATGGGAATGGG	CATGGCACGCAT	GTGGCCGGGACG
	ATTGCTGCTTTA	AACAATTCGATT	GGCGTTCTTGGC	GTAGCGCCGAGC	GCGGAACTATAC
20	GCTGTTAAAGTA	TTAGGGGCGAGC	GGTTCAGGTTTCG	GTCAGCTCGATT	GCCCAAGGATTG
	GAATGGGCAGGG	AACAATGGCATG	CACGTTGCTAAT	TTGAGTTTAGGA	AGCCCTTCGCCA
			XbaI		
	AGTGCCACACTT	GAGCAAGCTGTT	AATAGCGCGACT	TCTAGAGGCGTT	CTTGTTGTAGCG
25	GCATCTGGGAAT	TCAGGTGCAGGC	TCAATCAGCTAT	CCGGCCCCTTAT	GCGAACGCAATG
	GCAGTCGGAGCT	ACTGACCAAAC	AACAACCGCGCC	AGCTTTTCACAG	TATGGCGCAGGG
	CTTGACATTGTC	GCACCAGGTGTA	AACGTGCAGAGC	ACATACCCAGGT	TCAACGTATGCC
		Clai			
	AGCTTAAACGGT	ACATCGATGGCT	ACTCCTCATGTT	GCAGGTGCAGCA	GCCCTTGTTAAA
30	CAAAAGAACCCA	TCTTGGTCCAAT	GTACAAATCCGC	AATCATCTAAAG	AATACGGCAACG

-35-

AGCTTAGGAAGC ACGAACTTGTAT GGAAGCGGACTT GTCAATGCAGAA GCGGCAACACGC

Stop  
TAA

1141

5

The same procedure as above was used for the cloning of the subtilisin 147 gene except that the DNA fragments were ligated into the plasmid pSX56 (also described in US serial No. 039,298 supra), which as indicated in Figure 2 instead of the xyn promotor harbours the xyl promotor. One clone was found harbouring a plasmid, pSX94, carrying the gene for subtilisin 147. The sequence for this gene is shown in table III below.

15

Table III

20

THE SUBTILISIN 147 GENE

Signal

	ATGAGACAAAGT	CTAAAAGTTATG	GTTTTGTCAACA	GTGGCATTGCTT	TTCATGGCAAAC
	1	Pro			
25	CCAGCAGCAGCA	GGCGGGGAGAAA	AAGGAATATTTG	ATTGTCGTCGAA	CCTGAAGAAGTT
		73			
	TCTGCTCAGAGT	GTCGAAGAAAGT	TATGATGTGGAC	GTCATCCATGAA	TTTGAAGAGATT
	CCAGTCATTCAT	GCAGAACTAACT	AAAAAAGAATTG	AAAAAATTAAAG	AAAGATCCGAAC
				Mature	
	GTAAAAGCCATC	GAAGAGAATGCA	GAAGTAACCATC	AGTCAAACGGTT	CCTTGGGGAATT
				280	
30	TCATTCATTAAT	ACGCAGCAAGCG	CACAACCGCGGT	ATTTTGGGTAAC	GGTGCTCGAGTC

35

-36-

GCTGTCCTTGAT ACAGGAATTGCT TCACACCCAGAC TTACGAATTGCA GGGGGAGCGAGC  
 TTTATTTCAAGC GAGCCTTCCTAT CATGACAATAAC GGACACGGAACT CACGTGGCTGGT  
 ACAATCGCTGCG TTAAACAATTCA ATCGGTGTGCTT GGTGTACGACCA TCGGCTGACTTG  
 5 TACGCTCTCAAA GTTCTTGATCGG AATGGAAGTGGT TCGCTTGCTTCT GTAGCTCAAGGA  
 ATCGAATGGGCA ATTAACAACAAC ATGCACATTATT AATATGAGCCTT GGAAGCACGAGT  
 GGTTCCTAGCACG TTAGAGTTAGCT GTCAACCGAGCA AACAAATGCTGGT ATTCTCTTAGTA  
 GGGGCAGCAGGT AATACGGGTTAGA CAAGGAGTTAAC TATCCTGCTAGA TACTCTGGTGGT  
 10 ATGGCGGTTGCA GCAGTTGATCAA AATGGTCAACGC GCAAGCTTCTCT ACGTATGGCCCA  
 GAAATTGAAATT TCTGCACCTGGT GTCAACGTAAAC AGCACGTACACA GGCAATCGTTAC  
 GTATCGCTTTCT GGAACATCTATG GCAACACCACAC GTTGCTGGAGTT GCTGCACTTGTTG  
 AAGAGCAGATAT CCTAGCTATACG AACAAACCAAATT CGCCAGCGTATT AATCAAACAGCA  
 15 ACGTATCTAGGT TCTCCTAGCCTT TATGGCAATGGA TTAGTACATGCT GGACGTGCAACA  
 Stop  
 CAATAA  
 1084

20

#### 6.2.2. GENERATION OF SITE-SPECIFIC MUTATIONS OF THE SUBTILISIN 309 GENE

Site specific mutations were performed by the method of  
 25 Morinaga et al. (Biotechnology, supra). The following  
 oligonucleotides were used for introducing the mutations:

##### a) Gly-195 Glu:

A 27-mer mismatch primer, Nor-237, which also  
 30 generates a novel SacI restriction site

-37-

5' CACAGTATGGGCGCAGGGCTTGACATTGTCGCACCAGG 3'  
 NOR-237 5' GTATGGCGCAGAGCTCGACATTTGTCGC 3'  
 SacI

5 b) Gly-195 Asp:

A 23-mer mismatch primer, NOR-323, which also  
 generates a novel BglII site

AT  
 5' CACAGTATGGGCGCAGGGCTTGACATTGTC 3'  
 10 3' CATACCGCGTCTAGAACTGTAAC 5'  
 BglII

c) Met-222 Cys:

A 24-mer mismatch primer, NOR-236

15 ClaI  
 5' AGCTTAAACGGTACATCGATGGCTACTCCTCATGTT 3'  
 NOR-236 5' ACGGTACATCGTGGCTACTCCTC 3'

d) Met-222 Ala:

A 22-mer mismatch primer, NOR-235

20 ClaI  
 5' AGCTTAAACGGTACATCGATGGCTACTCCTCATGTT 3'  
 NOR-235 5' CGGTACATCGGCGGCTACTCCT 3'

25 Both of these primers destroy the unique ClaI site

e) Ser-153 Ala:

An 18-mer mismatch primer, NOR-324, which also  
 generates a novel PvuII site

30 G  
 5' CTTGTAGCGGCATCTGGAATTCAGGT 3'  
 NOR-324 3' CATCGCCGTCGACCCTTA 5'  
 PvuII

35

-38-

f) Asn-218 Ser:

A 23-mer mismatch primer, NOR-325, which also generates a novel MspI site

5 TC  
 5' TATGCCAGCTTAAACGGTACATCGATG 3'  
 NOR-324 3' TACGGTCGAATAGGCCATGTAGC 5'  
 MspI

g) Thr-71 Asp:

A 23-mer mismatch primer, NOR-483,

GAC  
 5' TGTGGCCCGGGACGATTGCTGCTT 3'  
 15 NOR-483 3' ACACCGGCCCCCTGTAACGACGAA 5'

h) Met-222 Cys and Gly-219 Cys:

A 32-mer mismatch, NOR-484,

T TGT  
 5' CAGCTTAAACGGTACATCGATGGCTACTCCTC 3'  
 20 219 222  
 NOR-484 3' GTCGAATTTGACATGTAGCACACGATGAGGAG 5'

i + j) Gly-195 Glu and Met-222 Ala or Met-222 Cys:

25 For these double mutants combinations of NOR-237 and NOR-235 or NOR-236 were performed by joining the single mutant DNA-fragments.

k) Ser-153 Ala and Asn-218 Ser:

30 A combination of NOR-324 and NOR-325 was performed in analogy with the above.

Gapped duplex mutagenesis was performed using the plasmid pSX93 as template. pSX93 is shown in Figure 3a and



-39-

3b, and is pUC13 (Vieira, J. and Messing, J.: 1982, Gene 19: 259-268) harbouring an 0.7 kb XbaI-HindIII fragment of the subtilisin 309 gene including the terminator inserted in the polylinker. The terminator and the HindIII site are not shown in Table II.

For the introduction of mutations in the N-terminal part of the enzyme the plasmid pSX119 was used. pSX119 is pUC13 harbouring an EcoRI-XbaI fragment of the subtilisin 309 gene inserted into the polylinker. The templates pSX93 and pSX119 thus cover the whole of the subtilisin 309 gene.

The mutations a), b), and e) were performed by cutting pSX93 with XbaI and ClaI as indicated in Figure 3a; c), d), f), and h) were performed by cutting pSX93 with XbaI and HindIII as indicated in Figure 3b.

Mutation g) was performed correspondingly in pSX119 by cutting with EcoRI and XbaI.

The double mutants i) and j) were produced by cutting the 0.7 kb Xba-HindIII fragment from a) partially with HgiAI (HgiAI also cuts in SacI, which was introduced by the mutation). This 180 bp XbaI-HgiAI fragment and the 0.5 kb HgiAI fragment from the c) and d) mutants, respectively, were ligated to the large HindIII-XbaI fragment from pSX93.

The double mutant k) was produced as above by combining mutants e) and f).

Subsequent to annealing, filling and ligation the mixture was used to transform *E. coli* MC 1000  $r^{-}m^{+}$ . Mutants among the transformants were screened for by colony hybridization as described in Vlasuk et al.: 1983, J.Biol.Chem., 258: 7141-7148 and in Vlasuk, G.P. and Inouye, S.: p. 292-303 in "Experimental Manipulation of Gene Expression" Inouye, M. (ed.) Academic Press, New York. The mutations were confirmed by DNA sequencing.

-40-

### 6.2.3. EXPRESSION OF MUTANT SUBTILISINS

Subsequent to sequence confirmation of the correct mutation the mutated DNA fragments were inserted into plasmid pSX92, which was used for producing the mutants.

5 Plasmid pSX92 is shown in Figure 4 and was produced by cloning the Sub 309 gene into plasmid pSX62 cut at ClaI, filled in with the Klenow fragment of DNA polymerase I, and cut with HindIII prior to the insertion of the fragments DraI-NheI and NheI-HindIII from the cloned Sub 309  
10 gene.

To express the mutants the mutated fragments (XbaI-ClaI, XbaI-HindIII, or EcoRI-XbaI) were excised from the appropriate mutation plasmid pSX93 or pSX119, respectively, and inserted into pSX92.

15 The mutated pSX92 was then used to transform B. subtilis strain DN497, which was then grown in the same medium and under the same conditions as used for the cloning of the parent gene.

After appropriate growth the mutated enzymes were  
20 recovered and purified.

### 6.2.4. OXIDATION STABILITY OF MUTANT SUBTILISINS

The mutants a) and d) were tested for their oxidation stability in 0.01 M peracetic acid after 20 minutes at 50°C and pH 7. The parent strain NCIB 10309 protease was used as  
25 reference.

The results are indicated in Table IV below, which presents the residual proteolytic activity in the heat treated samples relative to samples untreated by oxidant or  
30 heat.

-41-

Table IV  
Oxidation Stability Towards Peracetic Acid

Enzyme	Residual Activity after 20 min. at 50°C	
	without oxidant	with oxidant
5 sub 309	89%	48%
mutant a	83%	45%
mutant d	92%	93%

10 It is concluded that mutant d (Met 222 to Ala) exhibits superior oxidation stability, relative to the parent enzyme and mutant a.

15 All the mutants except g) and h) have also been tested qualitatively in 100 - 500 ppm hypochlorite at room temperature and 35°C, pH 6.5 and 9.0, for from 15 minutes to 2 hours.

20 These tests showed that mutants c), d), i), and j) (all Met-222) could resist 3 - 5 times more hypochlorite than the other mutants.

When tested in a liquid detergent of the usual built type it was found that mutant f) exhibited superior stability compared to both the other mutants and the "parent" enzyme.

#### 25 6.2.5. PROTEOLYTIC ACTIVITY OF MUTANT SUBTILISINS

The proteolytic activity of various mutants was tested against casein as protein substrate, according to methods detailed supra. The results are presented in Table V.

30 From the table it is seen that mutant a) exhibits enhanced activity compared to the parent. It is also seen that the Met-222 mutants have lower activity than the parent, but due to their improved oxidation stability their

-42-

application in detergent compositions containing oxidants is not precluded.

TABLE V

5

Proteolytic Activity of Mutant Subtilisins

	Mutant	Relative Activity
10	None	100
	a)	120
	b)	100
	c)	30
	d)	20
15	e)	100
	f)	100
	i)	20
	j)	30

20

6.2.6. WASHABILITY OF MUTANT SUBTILISINS

The washability of various mutants was tested against spinach juice according to methods detailed supra. The results are presented in Table VI.

25

From the table it is seen that all of the tested mutants exhibited an improved washing ability compared to the parent enzyme, and that mutants c), d), i), and j) are markedly superior.

30

35

-43-

TABLE VIWashing Ability of Mutant

5	Mutant	$\Delta R$	
		Concentration	(CFU/l)
		0.05	0.1
	none	14.4	20.4
10	a)	18.8	21.5
	b)	16.9	19.7
	c)	21.8	23.8
	d)	22.2	23.4
	e)	15.4	21.8
15	f)	16.6	19.3
	i)	21.6	22.1
	j)	20.6	22.6

95% confidence interval:  $\pm 0.9$ 

20

6.2.7. THERMOSTABILITY OF MUTANT SUBTILISINS

The thermostability of mutant f) was tested against the wild type enzyme by using the washability test at 40°C and 60°C, respectively. The results are shown in Table VII.

25

From the table it is seen that mutant f) at 60°C shows a much improved washability compared to the wild type enzyme, whereas at 40°C the washability of mutant f) is only slightly better than wild type enzyme.

30

35

-44-

TABLE VIIWashability at Different Temperatures

5	Mutant	<u>ΔR</u>	
		Concentration	(CPU/l)
		0.05	0.1
	none (40°C)	14.4	20.4
10	f) (40°C)	16.6	19.3
	none (60°C)	15.1	24.9
	f) (60°C)	30.4	31.3
	95% confidence interval $\pm 0.9$ (40°C) and $\pm 0.7$ (60°)		

6.3. DISCUSSION

15 Subtilisin genes were cloned from the 147 and 309 variants of the bacterium Bacillus lentus, and the cloned genes were sequenced. By comparing the deduced amino acid sequences of subtilisins 147 and 309 one with the other and  
 20 with sequences of other subtilisins, sites which, upon mutation, might alter the physical properties of the parent enzyme were identified. Site-directed mutagenesis was used to generate mutations at several of these sites in the subtilisin 309 gene. The resulting mutant enzymes were then  
 25 expressed in a Bacillus strain, and tested against various physical and chemical parameters. Several of the mutants were shown to have improved stability to oxidation, increased proteolytic ability, or improved washability when compared with parent subtilisin 309 enzymes. These mutants  
 30 exhibit properties desirable in enzymes comprised in detergent compositions.

WHAT IS CLAIMED IS:

1. A mutant subtilisin enzyme in which the amino acid  
5 residue at one or more of the positions:  
6, 9, 11-12, 19, 25, 36-38, 53-59, 67, 71, 89, 111, 115,  
120, 121-122, 124, 128, 131, 140, 153, 154, 156, 158-166,  
168, 170, 172, 175, 180, 182, 186, 187, 191, 194, 195, 199,  
218, 219, 226, 234-238, 241, 260-262, 265, 268, or 275  
10 is changed by substitution with another amino acid residue,  
or insertion or deletion of one or more amino acid residues.
2. The mutant subtilisin enzyme of claim 1 in which  
one or more amino acid residues are inserted at one or more  
15 of the positions: 36, 56, 159, or 164-166.
3. The mutant subtilisin enzyme of claim 1 in which  
the residue at position 6 is substituted with tyrosine.
- 20 4. The mutant subtilisin enzyme of claim 1 in which  
the residue at position 67 is substituted with glutamic acid  
or aspartic acid.
- 25 5. The mutant subtilisin enzyme of claim 1 in which  
the residue at position 68 is substituted with cysteine or  
methionine.
- 30 6. The mutant subtilisin enzyme of claim 1 in which  
the residue at position 71 is substituted with aspartic acid  
or glutamic acid.
7. The mutant subtilisin enzyme of claim 1 in which  
the residue at position 153 is substituted with alanine.

-46-

8. The mutant subtilisin enzyme of claim 1 in which the residue at position 168 is substituted with alanine.

9. The mutant subtilisin enzyme of claim 1 in which the residue at position 170 is substituted with tyrosine.

10. The mutant subtilisin enzyme of claim 1 in which the residue at position 175 is substituted with isoleucine.

11. The mutant subtilisin enzyme of claim 1 in which the residue at position 195 is substituted with glutamic acid or aspartic acid.

12. The mutant subtilisin enzyme of claim 1 in which the residue at position 218 is substituted with serine.

13. The mutant subtilisin enzyme of claim 1 in which the residue at position 219 is substituted with methionine.

14. The mutant subtilisin enzyme of claim 1 in which the residue at position 275 is substituted with glutamine.

15. The mutant subtilisin enzyme of claim 1 in which the residue at position 19 is substituted with glycine and the residue at position 219 is substituted with cysteine.

16. The mutant subtilisin enzyme of claim 1 in which the residue at position 153 is substituted with alanine and the residue at position 218 is substituted with serine.

17. The mutant subtilisin enzyme of claim 1 in which the residue at position 195 is substituted with glutamic



-47-

acid and the residue at position 222 is substituted with alanine or cysteine.

18. The mutant subtilisin enzyme of claim 1 in which  
5 the residue at position 219 is substituted with cysteine and  
the residue at position 222 is substituted with cysteine.

19. The mutant subtilisin enzyme of claim 1 in which  
the parent enzyme comprises an amino acid sequence  
10 substantially as depicted in Table I(c).

20. The mutant subtilisin enzyme of claim 1 in which  
the parent enzyme comprises an amino acid sequence  
substantially as depicted in Table I(d).

15 21. The mutant subtilisin enzyme of claim 1 in which  
the parent enzyme comprises an amino acid sequence  
substantially as depicted in Table I(e).

20 22. A mutant subtilisin enzyme 309 comprising the  
amino acid sequence substantially as depicted in Table I(a).

23. The mutant subtilisin enzyme 309 of Claim 22 in  
which the amino acid residue at one or more of the following  
25 positions is substituted by another amino acid residue: 6,  
9, 11-12, 19, 25, 36-38, 53-59, 67, 71, 89, 104, 111, 115,  
120, 121-122, 124, 128, 131, 140, 153-166, 168, 169-170,  
172, 175, 180, 182, 186, 187, 191, 194, 195, 199, 218, 219,  
222, 226, 234-238, 241, 260-262, 265, 268, or 275.

30 24. The mutant subtilisin enzyme 309 of Claim 22 in  
which an amino acid is inserted at one or more of the  
following positions: 36, 56, 159 or 164-166.

35

-48-

25. The mutant subtilisin enzyme 309 of Claim 23 in which the tryptophan residue at position 6 is substituted with tyrosine.

5 26. The mutant subtilisin enzyme 309 of Claim 23 in which the histidine residue at position 67 is substituted with glutamic acid or aspartic acid.

10 27. The mutant subtilisin enzyme 309 of Claim 23 in which the valine residue at position 68 is substituted with cysteine or methionine.

15 28. The mutant subtilisin enzyme 309 of Claim 23 in which the threonine residue at position 71 is substituted with aspartic acid or glutamic acid.

29. The mutant subtilisin enzyme 309 of Claim 23 in which the serine residue at position 153 is substituted with alanine.

20 30. The mutant subtilisin enzyme 309 of Claim 23 in which the proline residue at position 168 is substituted with alanine.

25 31. The mutant subtilisin enzyme 309 of Claim 23 in which the arginine residue at position 170 is substituted with tyrosine.

30 32. The mutant subtilisin enzyme 309 of Claim 23 in which the methionine residue at position 175 is substituted with isoleucine.

-49-

33. The mutant subtilisin enzyme 309 of Claim 23 in which the glycine residue at position 195 is substituted with glutamic acid or aspartic acid.

5 34. The mutant subtilisin enzyme 309 of Claim 23 in which the asparagine residue at position 218 is substituted with serine.

10 35. The mutant subtilisin enzyme 309 of Claim 23 in which the glycine residue at position 219 is substituted with methionine.

15 36. The mutant subtilisin enzyme 309 of Claim 23 in which the methionine residue at position 222 is substituted with cysteine or alanine.

37. The mutant subtilisin enzyme 309 of Claim 23 in which the arginine residue at position 275 is substituted with glutamine.

20 38. The mutant subtilisin enzyme 309 of Claim 23 in which the arginine residue at position 19 is substituted with glycine and the glycine residue at position 219 is substituted with cysteine.

25 39. The mutant subtilisin enzyme 309 of Claim 23 in which the serine residue at position 153 is substituted with alanine and the asparagine residue at position 218 is substituted with serine.

30 40. The mutant subtilisin enzyme 309 of Claim 23 in which the glycine residue at position 195 is substituted

-50-

with glutamic acid and the methionine residue at position 222 is substituted with alanine or cysteine.

41. The mutant subtilisin enzyme 309 of Claim 23 in which the glycine residue at position 219 is substituted with cysteine and the methionine residue at position 222 is substituted with cysteine.

42. A mutant subtilisin enzyme 147 comprising the amino acid sequence substantially as depicted in Table I(b).

43. The mutant subtilisin enzyme 147 of Claim 42 in which the amino acid residue at one of the following positions is substituted by another amino acid residue: 6, 9, 11-12, 19, 25, 36-38, 53-59, 67, 71, 89, 111, 115, 120, 121-122, 124, 128, 131, 140, 153-166, 168, 169-170, 172, 175, 180, 182, 186, 187, 191, 194, 195, 199, 218, 219, 222, 226, 234-238, 241, 260-262, 265, 268 or 275.

44. The mutant subtilisin enzyme 147 of Claim 42 in which an amino acid is inserted at one of the following positions: 1, 36, 56, 159 or 164-166.

45. The mutant subtilisin enzyme 147 of Claim 43 in which the tryptophan residue at position 6 is substituted with tyrosine.

46. The mutant subtilisin enzyme 147 of Claim 43 in which the histidine residue at position 67 is substituted with glutamic acid or aspartic acid.

-51-

47. The mutant subtilisin enzyme 147 of Claim 43 in which the valine residue at position 68 is substituted with cysteine or methionine.

5 48. The mutant subtilisin enzyme 147 of Claim 43 in which the threonine residue at position 71 is substituted with aspartic acid or glutamic acid.

10 49. The mutant subtilisin enzyme 147 of Claim 43 in which the alanine residue at position 153 is substituted with serine.

15 50. The mutant subtilisin enzyme 147 of Claim 43 in which the proline residue at position 168 is substituted with alanine.

51. The mutant subtilisin enzyme 147 of Claim 43 in which the arginine residue at position 170 is substituted with tyrosine.

20 52. The mutant subtilisin enzyme 147 of Claim 43 in which the methionine residue at position 175 is substituted with isoleucine.

25 53. The mutant subtilisin enzyme 147 of Claim 43 in which the glutamic acid residue at position 195 is substituted with glycine or aspartic acid.

30 54. The mutant subtilisin enzyme 147 of Claim 43 in which the serine residue at position 218 is substituted with asparagine.

-52-

55. The mutant subtilisin enzyme 147 of Claim 43 in which the glycine residue at position 219 is substituted with methionine.

5 56. The mutant subtilisin enzyme 147 of Claim 43 in which the methionine residue at position 222 is substituted with cysteine or alanine.

10 57. The mutant subtilisin enzyme 147 of Claim 43 in which the glutamine residue at position 275 is substituted with arginine.

15 58. The mutant subtilisin enzyme 147 of Claim 43 in which the arginine residue at position 19 is substituted with glycine and the glycine residue at position 219 is substituted with cysteine.

20 59. The mutant subtilisin enzyme 147 of Claim 43 in which the alanine residue at position 153 is substituted with serine and the serine residue at position 218 is substituted with asparagine.

25 60. The mutant subtilisin enzyme 147 of Claim 43 in which the glutamic acid residue at position 195 is substituted with glycine and the methionine residue at position 222 is substituted with alanine or cysteine.

30 61. The mutant subtilisin enzyme 147 of Claim 43 in which the glycine residue at position 219 is substituted with cysteine and the methionine residue at position 222 is substituted with cysteine.

62. A recombinant DNA molecule comprising all or part of a nucleotide sequence coding for a subtilisin enzyme comprising an amino acid sequence substantially as depicted in Table I(a).

5

63. A recombinant DNA molecule comprising all or part of a nucleotide sequence coding for a subtilisin enzyme comprising an amino acid sequence substantially as depicted in Table I(b).

10

64. A recombinant DNA molecule comprising all or part of a nucleotide sequence coding for the subtilisin 309 enzyme gene substantially as depicted in Table II.

15

65. A recombinant DNA molecule comprising all or part of a nucleotide sequence coding for the subtilisin 147 enzyme gene substantially as depicted in Table III.

20

66. The recombinant DNA molecule of claim 62 or 64 in which the nucleic acid sequence has been altered such that the corresponding amino acid residue at one or more of the positions:

6, 9, 11-12, 19, 25, 36-38, 53-59, 67, 71, 89, 104, 111, 115, 120, 121-122, 124, 128, 3, 40, 53-166, 168, 169-170, 172, 175, 180, 182, 186, 187, 191, 194, 195, 199, 218, 219, 222, 226, 234-238, 241, 260-262, 265, 268 or 275

25

is changed by substitution with another amino acid residue, or insertion or deletion of one or more amino acid residues.

30

67. The recombinant DNA molecule of claim 63 or 65 in which the nucleic acid sequence has been altered such that the corresponding amino acid residue at one or more of the positions:

35

-54-

6, 9, 11-12, 19, 25, 36-38, 53-59, 67, 71, 89, 104, 111, 115, 120, 121-122, 124, 128, 3, 40, 53-166, 168, 169-170, 172, 175, 180, 182, 186, 187, 191, 194, 195, 199, 218, 219, 222, 226, 234-238, 241, 260-262, 265, 268 or 275.  
5 is changed by substitution with another amino acid residue, or insertion or deletion of one or more amino acid residues.

68. The recombinant DNA molecule of claim 62 or 64 in which the nucleic acid sequence has been altered so as to  
10 insert one or more amino acid residues at one or more of the positions: 36, 56, 159, or 164-166.

69. The recombinant DNA molecule of claim 63 or 65 in which the nucleic acid sequence has been altered so as to  
15 insert one or more amino acid residues at one or more of the positions: 36, 56, 159, or 164-166.

70. Use of a recombinant DNA molecule of any of the claims 62 to 69 for the production of a subtilisin enzyme.  
20

25

30

35



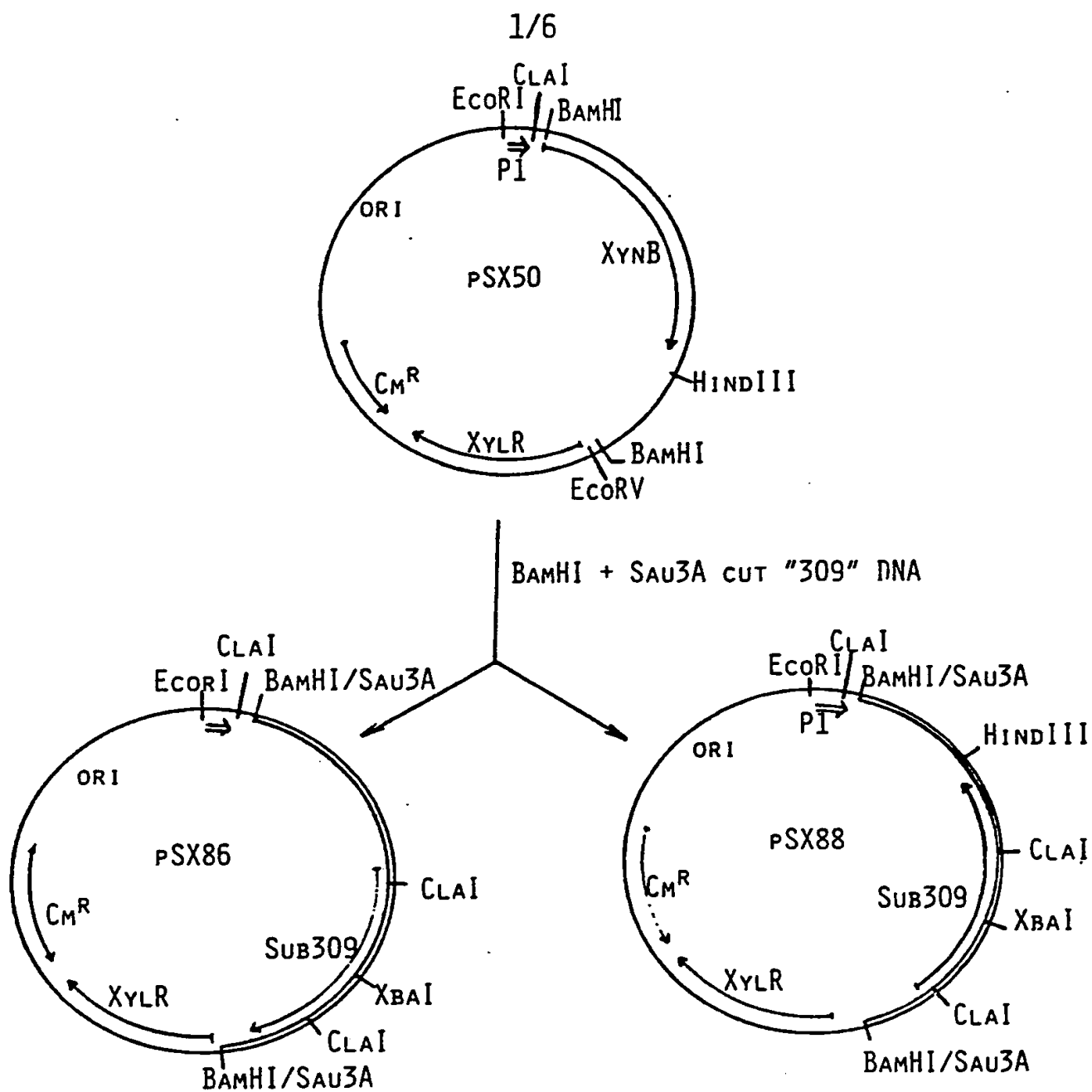


FIG. 1

2/6

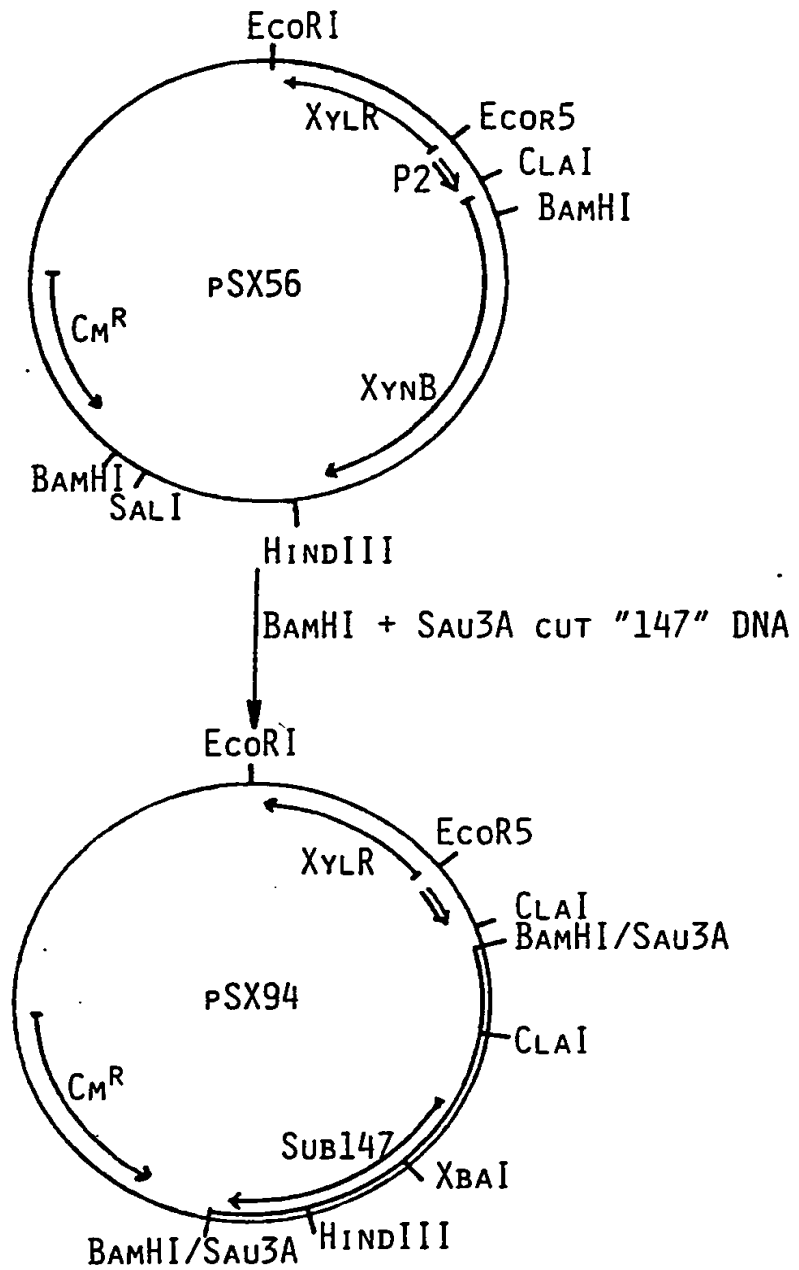


FIG. 2

3/6

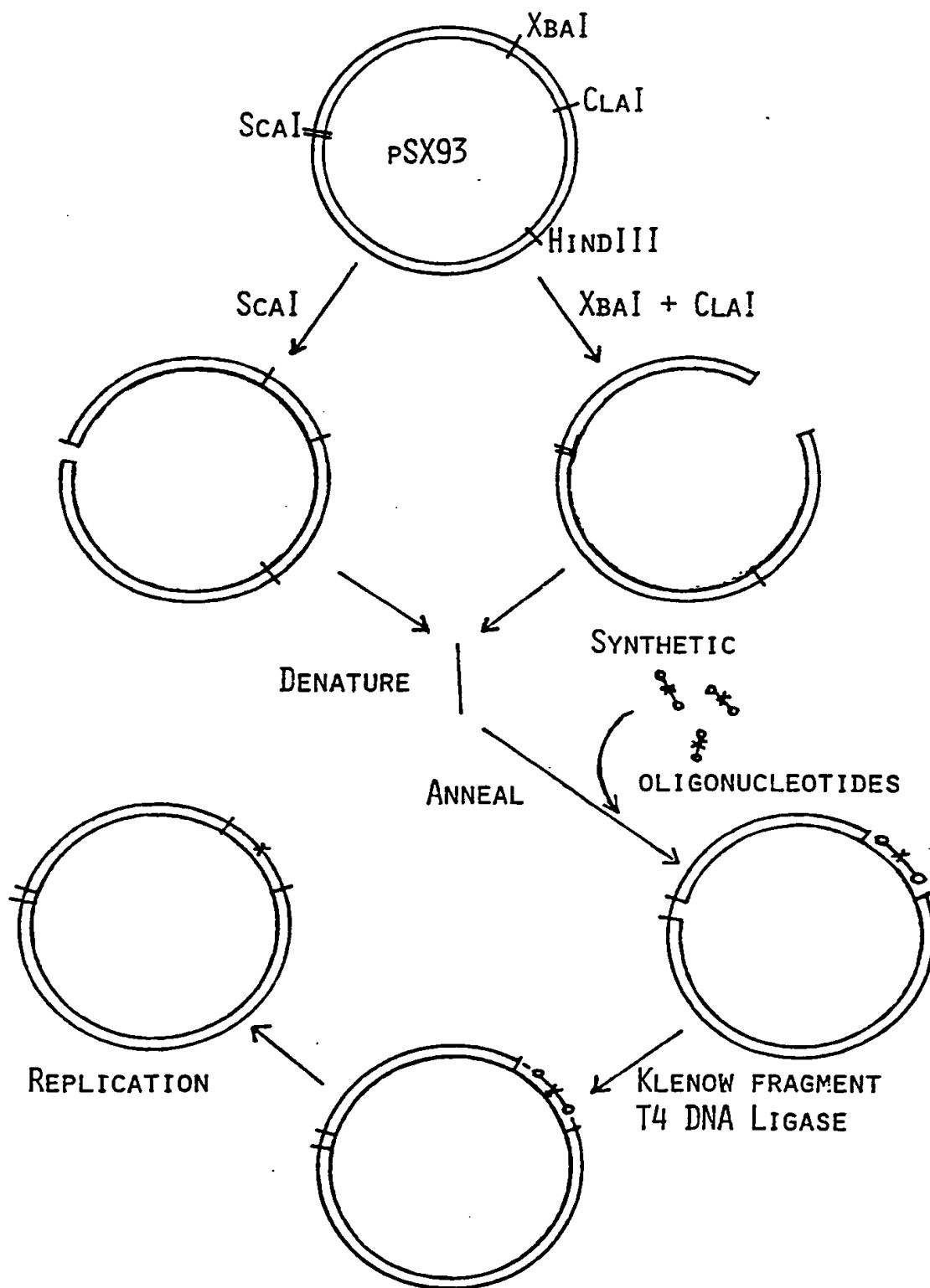


FIG. 3A

4/6

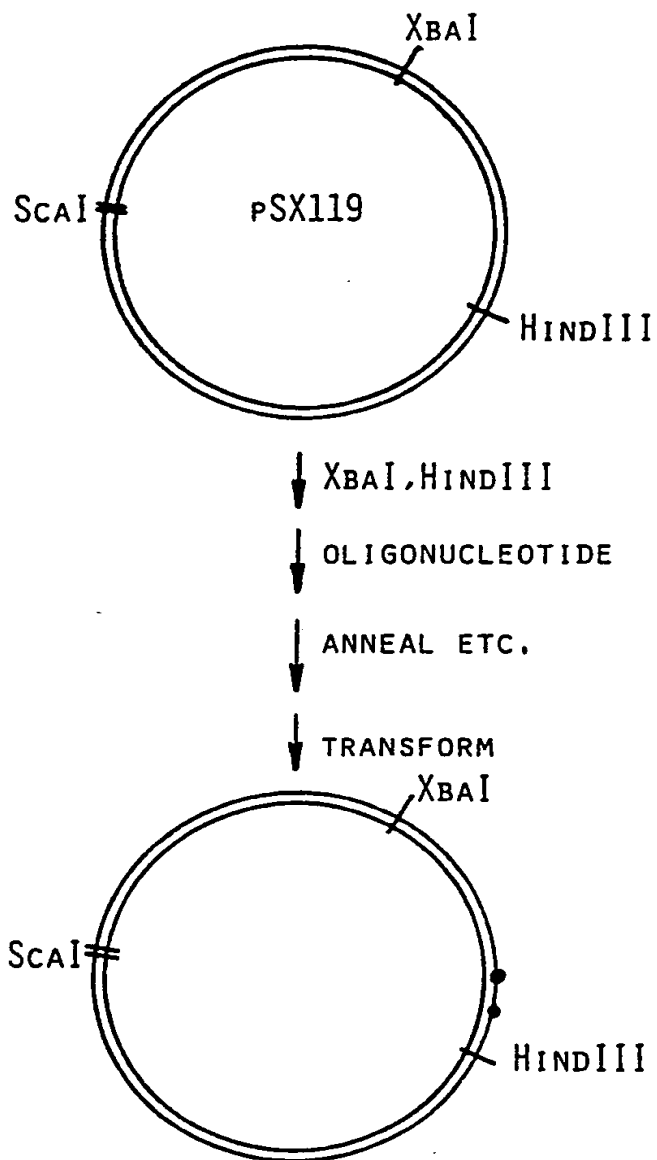


FIG. 3B

5/6

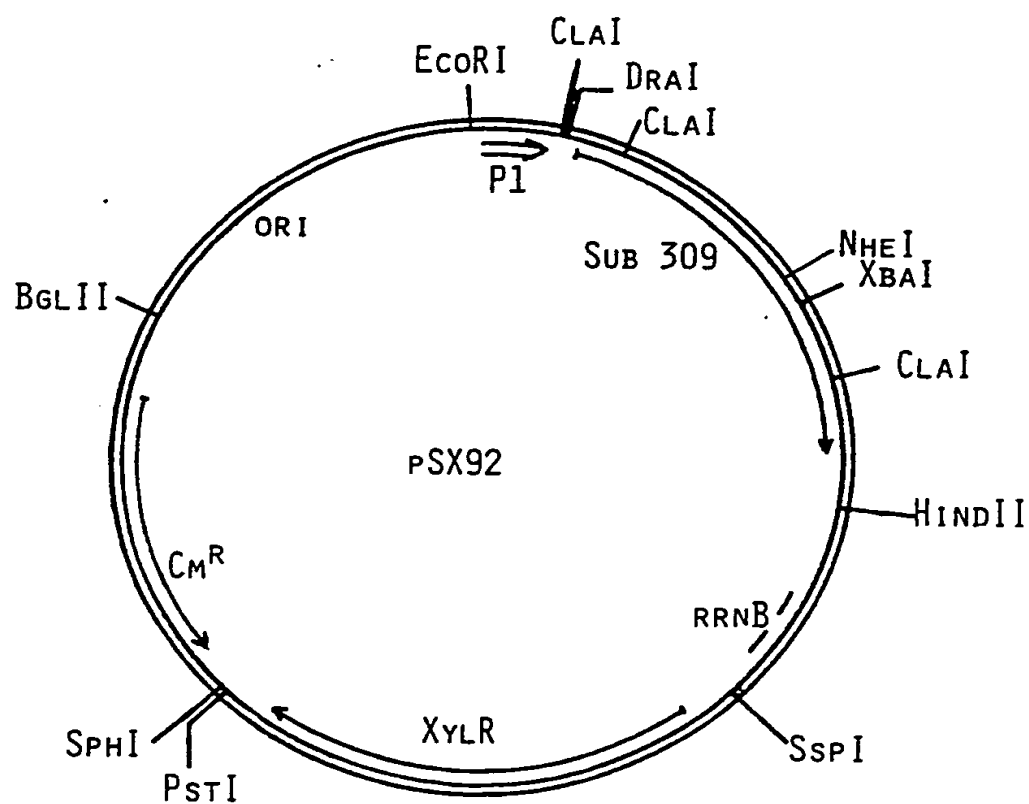


FIG. 4

6/6

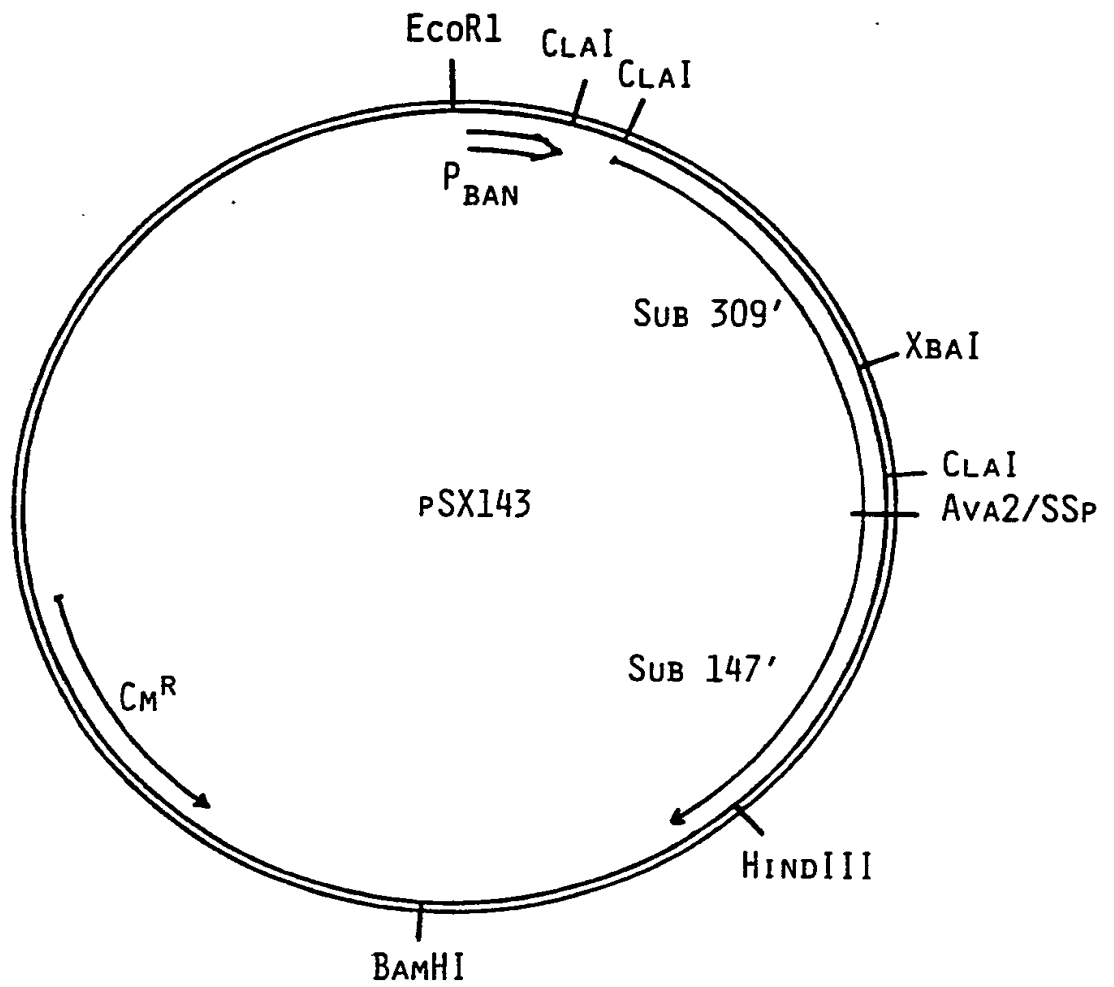


FIG. 5

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim numbers 1-70, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claims are found to be partly unsearchable because the scope of the claims is so broad. Due to the very large number of variable positions in the amino acid chain and in the choice of amino acids in each of these positions and because the general problem underlying the invention is unclear a full evaluation of the relevance of the state of the art literature has not been made.

3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 8.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	ington, D.C., 1883-) 1986, 233(4764), 659-63 (Eng).	3-22, 25-42, 45-65, 70
X	Chemical abstracts, Vol 105 (1986) abstract No 93546g, Chim.Oggi 1986, (3), 29-30 (Eng).	1, 2, 23, 24, 43, 44, 66-69
Y		3-22, 25-42, 45-65, 70
X	Chemical abstracts, Vol 108 (1988) abstract No 18427c, Proc.Natl.Acad. Sci. U.S.A. 1987, 84(15), 5167-71 (Eng).	1, 23, 43, 66, 67
Y		2-22, 24-42, 44-65, 68-70
A	Chemical abstracts, Vol 104 (1986) abstract No 221346k, J.Biol.Chem. 1986, 261(14), 6564-70 (Eng).	
A	Chemical abstracts, Vol 108 (1988) abstract No 108498f, Kollman, Peter; Rao, Shashidhar et al, Symp.Mol. Cell.Biol., New Ser. 1987, 69(Protein Struct., Folding, Des. 2), 215-25 (Eng).	
A	Chemical abstracts, Vol 108 (1988) abstract No 90970s, Protein Eng. 1987, 1(4), 319-24 (Eng).	
A	Chemical abstracts, Vol 107 (1987) abstract No 19936j, J.Mol.Biol. 1987, 193(4), 803-13 (Eng).	



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**